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13. ABSTRACT (Maximum 200 Words)

Neoangiogenesis process involves complex genetic expression alterations in endothelial cells. We performed SAGE analysis on purified endothelial cells from two freshly resected breast carcinoma and one normal breast tissue, finding that the expression of HEYL, a basic helix-loophelix (bHLH) transcription repressor, is consistently higher in the breast cancer libraries compared to normal breast tissue. Our in situ hybridization analysis using single sections and multi-tissue arrays validated the SAGE results.

To investigate the effect of HEYL on endothelial cells, we infected human umbilical vein endothelial cell (HUVEC) using adenovirus expressing HEYL. We found that the expression of HEYL can increase HUVEC proliferation. Under serum starvation, the cells expressing HEYL showed strong anti-apoptosis ability compared to control cells. In addition, the HEYL expressing cells elongated 3 days after infection and showed actin rearrangement. These elongated cells have increased invasion ability in Boyden chamber assay. We found that PI-3 kinase signal transduction pathway involved in the cell invasion, since PI-3 kinase inhibitor can effectively block the invasion.

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Body

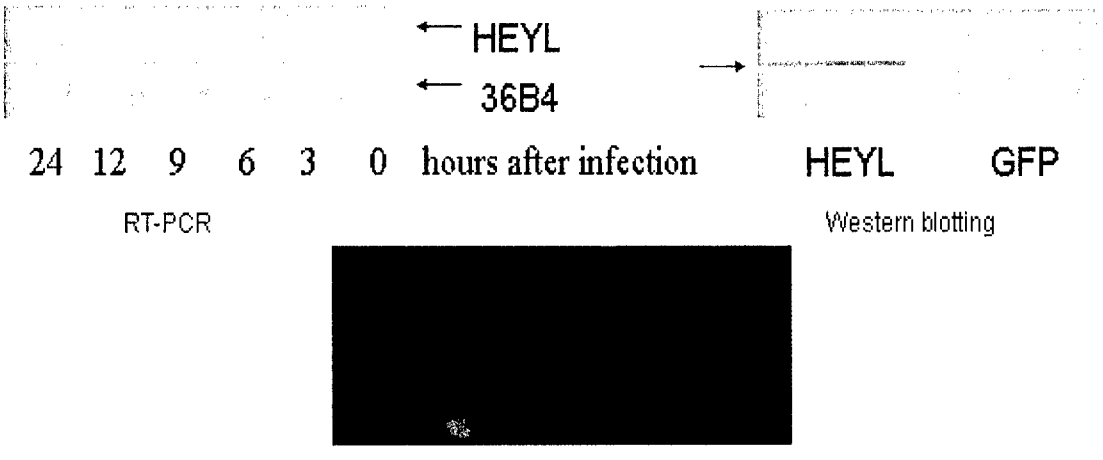
1. **Investigate the angiogenesis role of HEYL in breast cancer *in vitro***

A: Generate adenovirus expressing HEYL (ref. 1)and anti-HEYL polyclonal antibody

It is very hard to transfect vector into endothelial cells using lipo-based transfection reagents such as genejammer and lipofectin. The transfection efficiency could be as low as 5%. So we tried to make adenovirus expressing HEYL and used the virus to infect Human Umbilical Vein Endothelial Cells (HUVEC). In our viral constructor, the expressions of HEYL and green fluorescent protein (GFP) are under the control of 2 separate CMV promoters so that we can detect the infection efficiency by checking the cell green fluorescence. We find that 100% infection efficiency can be reached in our system.

We also generate anti-HEYL polyclonal antibody by injecting HEYL sequence-specific peptide into rabbit. Western blotting showed that this antibody can recognize a 40kd band only in HEYL expressing cell lysates and it can be blocked by HEYL sequence-specific peptide, suggesting its specificity to recognize HEYL.

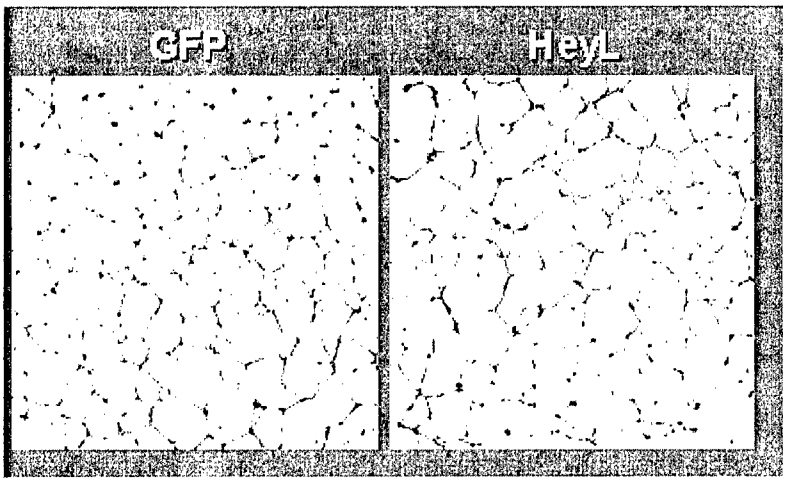
We used the virus to infect HUVEC and check HEYL mRNA expression by RT-PCR at 0,3,6,9 and 24 hours. We found HEYL was expressed as early as 3 hours after infection and sufficient expression persisted through 24 hours. Western blotting showed HEYL protein expression in HUVEC infected by adenovirus expressing HEYL but not in HUVEC infected by adenovirus expressing GFP. Immunofluorescence (IF) staining show HEYL localization in nucleus.



IF staining: HEYL-red, Nucleus-Blue

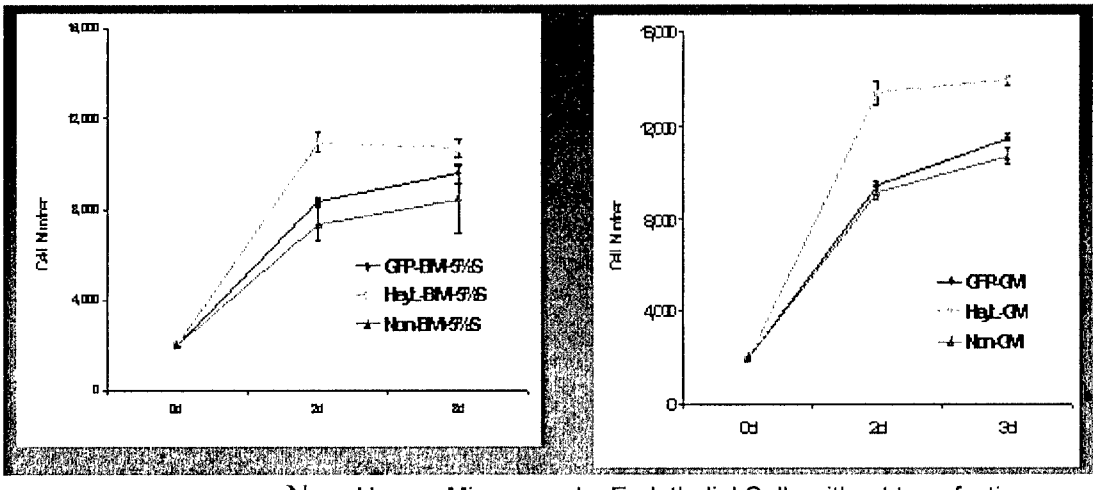
B: Culture HUVEC on Matrigel

HUVEC infected by adenovirus expressing HEYL or GFP were plated on Matrigel for 24 hours. We found both of them can form network structure. There was no difference in network density.



C: Proliferation assay

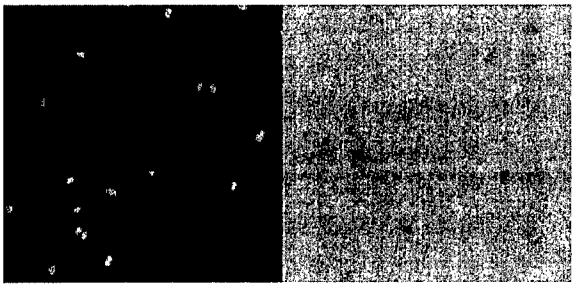
We infected Human Microvascular Endothelial Cells with adenovirus expressing HEYL or GFP. The cell numbers were counted for 3 days. The expression of HEYL increased cell proliferation in both basal medium plus 5% serum (BM-5%S) and growth medium (GM) which contains other growth factors. (ref. 2)



Non: Human Microvascular Endothelial Cells without transfection

D: Apoptosis assay

We found that HEYL-expressing HUVEC showed significant anti-apoptosis under the stress of serum and growth factors withdraw. HUVEC infected by adenovirus expressing HEYL or GFP were plated on cell culture dish and grew in basal medium for 3 days. Terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) assays labeled apoptotic cells *in situ*. (ref. 2)



GFP HEYL

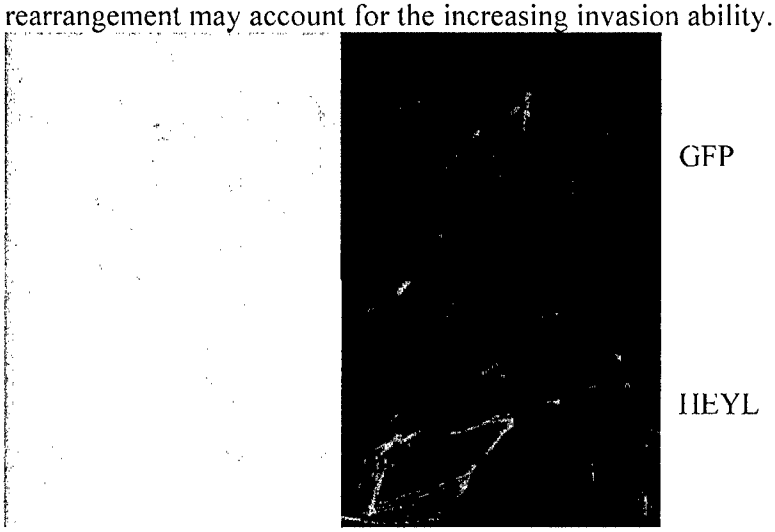
E: Invasion assay

HEYL or GFP adenovirus infected HUVEC were incubated in basal medium and plated on upper chamber of Boyden chamber. The normal growth media were put in the bottom chamber as chemoattractant. After 36 hours, the invasion cells invaded through Matrigel and reached the bottom of membrane. Those cells were blue-dye stained. We found that HEYL expression cells have stronger invasion ability.

GFP HEYL

F: Morphology change

We found HEYL expression cells became elongated compare to control cells. And actin rearrangement was shown by red phalloidin staining. This morphology change and actin rearrangement may account for the increasing invasion ability.

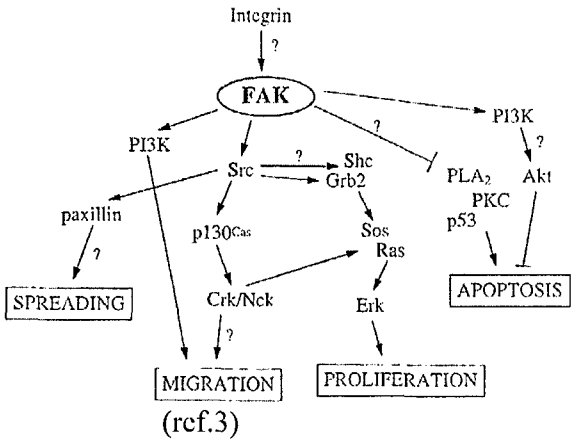


GFP

HEYL

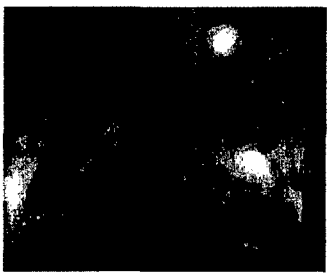
G: Possible molecular mechanism for HUVEC phenotypic changes

The above phenotypic changes can be explained by the activation of Focal Adhesion Kinase(FAK). (ref. 3) As shown in the figure, FAK activation can lead to anti-apoptois, proliferation and invasion. Therefore, we checked active form of FAK(FAK^{Y397}) by western blot and Immunofluorescence staining.

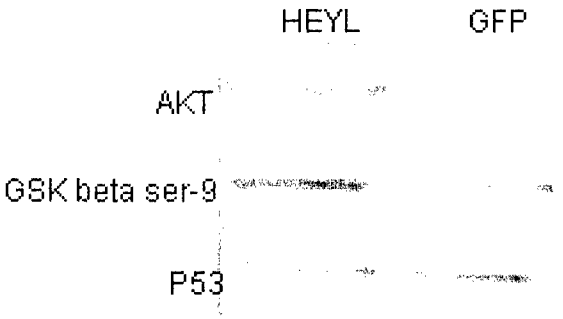


(ref.3)

HEYL GFP



Western blotting show FAK^{Y397} expression was upregulated in HEYL infected cells. And IF staining show this active FAK was strongly stained at cytoplasm membrane. We also checked the expression of molecules that are downstream of FAK signal transduction pathway and found AKT and GSK beta ser-9 expression were upregulated while p53 was not changed, suggesting the anti-apoptosis ability of HEYL expressing HUVEC is not mediated by P53.



Since AKT is activated by PI-3 kinase, we used PI-3 kinase inhibitor to block its activity. We found PI-3 kinase inhibitor can effectively block the invasion of HEYL expressing HUVEC, indicating that PI-3 kinase and AKT signal transduction pathway involved in the cell invasion.

2. Generating transgenic mice and studying the function of HEYL *in vivo*

We had planned to make 2 transgenic mice that express HEYL in all vasculature or in breast tissue endothelial cells. (ref. 4, 5) This involves complicated vectors development. Fortunately, all the vectors are now ready. We will inject these vectors into mouse ES cells. Hopefully, we will get the transgenic mice soon.

KEY RESEARCH ACCOMPLISHMENT

- Make adenovirus expressing HEYL and get satisfactory HEYL expression
- Generate anti-HEYL polyclonal antibody
- HEYL expression can increase cell proliferation in both basal medium plus 5% serum and growth medium
- HEYL expression can provide strong anti-apoptosis under stress of serum starvation
- HEYL expression can increase cell invasion
- Cells elongate and actin rearranged upon HEYL expression
- FAK activation and PI-3 kinase, AKT signal transduction pathway may be the molecular mechanism for these phenotypic changes *in vitro*
- All the vectors required for transgenic mice generation are made. ES cell injection will be done soon.

REPORTABLE OUTCOMES

paper: ALTERATIONS IN VASCULAR GENE EXPRESSION IN INVASIVE BREAST CARCINOMA.

CANCER RES. 2004 NOV 1;64(21):7857-66

CONCLUSION

Neoangiogenesis plays an important role in breast cancer development. Growth and formation of capillary blood vessels within a solid tumor are associated with tumor growth, metastasis and distant colonization. The angiogenic process involves complex genetic expression alterations in endothelial cells. To systematically search for endothelial genes that play an important angiogenic role in breast cancer, we performed SAGE analysis on purified endothelial cells from two freshly resected breast carcinoma and one normal breast tissue, finding that the expression of HEYL, a basic helix-loop-helix (bHLH) transcription repressor, is consistently higher in the breast cancer libraries compared to normal breast tissue. Our in situ hybridization analysis using single sections and multi-tissue arrays validated the SAGE results.

To investigate the effect of HEYL on endothelial cells, we infected human umbilical vein endothelial cell (HUVEC) using adenovirus expressing HEYL. We found that the expression of HEYL can increase HUVEC proliferation. Under serum starvation, the cells expressing HEYL showed strong anti-apoptosis ability compared to control cells. In addition, the HEYL expressing cells elongated 3 days after infection and showed actin rearrangement. These elongated cells have increased invasion ability in Boyden chamber assay. We found that PI-3 kinase signal transduction pathway involved in the cell invasion, since PI-3 kinase inhibitor can effectively block the invasion.

In summary, we identified the gene HEYL overexpressing in breast cancer endothelial cells by performing SAGE analysis on isolated breast cancer endothelial cells. We have shown that HEYL can affect various aspects of HUVEC including proliferation, anti-apoptosis and migration. In future studies, using HEYL transgenic mice and HEYL knockout mice, we will perform crosses with cancer prone HER2/neu transgenic mice, to test whether the susceptibility of these mice to tumorigenesis is modified in HEYL overexpression or underexpression condition. Thus, in a systematic way, we will study the role of HEYL in breast cancer progression.

REFERENCE

1. He TC, Zhou S, da Costa LT, Yu J, Kinzler KW, Vogelstein B. A simplified system for generating recombinant adenoviruses, Proc Natl Acad Sci U S A. 1998 Mar 3;95(5):2509-14
2. Parker BS, Argani P, Cook BP, Liangfeng H, Chartrand SD, Zhang M, Saha S, Bardelli A, Jiang Y, St Martin TB, Nacht M, Teicher BA, Klinger KW, Sukumar S, Madden SL. Alterations in vascular gene expression in invasive breast carcinoma, Cancer Res. 2004 Nov 1;64(21):7857-66
3. Cary LA, Guan JL. Focal adhesion kinase in integrin-mediated signaling. Front Biosci. 1999 Jan 15;4:D102-13.
4. Phelan A, Elliott G, O'Hare P. Intercellular delivery of functional p53 by the herpesvirus protein VP22. Nat Biotechnol 1998 May;16(5):440-3
5. Bennett RP, Dalby B, Guy PM. Protein delivery using VP22. Nat Biotechnol 2002 Jan;20(1):20

APPENDICES: Published paper

[CANCER RESEARCH 64, 7857–7866, November 1, 2004]

Alterations in Vascular Gene Expression in Invasive Breast Carcinoma

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ABSTRACT

The molecular signature that defines tumor microvasculature will likely provide clues as to how vascular-dependent tumor proliferation is regulated. Using purified endothelial cells, we generated a database of gene expression changes accompanying vascular proliferation in invasive breast cancer. In contrast to normal mammary vasculature, invasive breast cancer vasculature expresses extracellular matrix and surface proteins characteristic of proliferating and migrating endothelial cells. We define and validate the up-regulated expression of VE-cadherin and osteonectin in breast tumor vasculature. In contrast to other tumor types, invasive breast cancer vasculature induced a high expression level of specific transcription factors, including SNAIL1 and HEYL, that may drive gene expression changes necessary for breast tumor neovascularization. We demonstrate the expression of HEYL in tumor endothelial cells and additionally establish the ability of HEYL to both induce proliferation and attenuate programmed cell death of primary endothelial cells *in vitro*. We also establish that an additional intracellular protein and previously defined metastasis-associated gene, *PRL3*, appears to be expressed predominately in the vasculature of invasive breast cancers and is able to enhance the migration of endothelial cells *in vitro*. Together, our results provide unique insights into vascular regulation in breast tumors and suggest specific roles for genes in driving tumor angiogenesis.

INTRODUCTION

A critical role has been established linking unchecked microvascular proliferation and tumor growth. One of the most studied cancers in relation to neovascularization, breast cancer has provided a paradigm for the role of angiogenesis in cancer (1). Defining the gene expression alterations associated with tumor-driven neovascularization may yield therapeutically important targets for cancer intervention. In recent years there have been technological advances allowing large-scale expression profiling of cancer. Such profiling, including microarray (2) and serial analysis of gene expression (3, 4), along with the recent sequencing of the human genome, have advanced our current understanding of the molecular pathways involved in cancer progression. Such studies would be helpful in breast cancer, because there is a lack of molecular markers that can allow for an accurate prediction of response to specific therapies or more precisely determine whether a tumor is likely to metastasize to distant organs (5, 6). Heterogeneity in primary breast tumors, containing widely varying quantity and makeup of surrounding stroma, have made it difficult to provide common gene expression profiles that could more precisely predict the invasive capacity of a tumor and determine whether specific treatments will be effective therapies.

Bulk tissue expression profiling may mask differential expression in specific cell types of the tumor, as recent findings reveal genetic alterations that occur in cells surrounding epithelial tumors (reviewed in ref. 7). Moreover, using whole tumors may also mask the profiles of metastatic epithelial cells, because only a small proportion may have an underlying metastatic potential (8). It is becoming evident that stromal cells and the extracellular matrix interact with tumor epithelium to influence cancer progression. Such an influence is evidenced by the fact that tumor cells grow and metastasize best at their orthotopic site (compared with ectopic sites), associated with marked differences in angiogenesis (reviewed in ref. 9). Because the stromal microenvironment is important (10 12), it is necessary to study the molecular consequences associated with the cross-talk between cell types to gain a more comprehensive understanding of tumor progression. Many genes have been found to be aberrantly expressed in tumor epithelium and more recently in the surrounding stroma (13), yet there is little information on the gene expression alterations that occur in the breast vascular endothelium that may ultimately promote angiogenesis and provide a route for tumor cell dissemination into the circulation.

Enhanced angiogenesis is associated with an increased risk of metastasis and poor prognosis in breast cancer (14, 15). Neo-angiogenesis is also required at the metastatic site allowing micrometastases to grow into macrometastatic lesions. Therefore, angiogenesis within metastases is a very desirable therapeutic target considering the mortality associated with distant metastasis in breast cancer. Profiling expression changes that occur in the vasculature of breast cancer will provide insight into the mechanisms underlying tumor vascular growth and also reveal attractive targets for antiangiogenic therapies.

Serial analysis of gene expression technology is a powerful technology that has been used for expression profiling of both specific cell types (16, 17) and bulk tumors, including primary breast tumors (18). Serial analysis of gene expression is an open gene expression platform providing analysis of the entire transcriptome with a quantitative, digital output. To date, expression profiling from cancer-associated, pure vascular-specific cells has been limited to a serial analysis of gene expression application on a single normal and tumor endothelial cell preparation from colon (16) and several normal brain and malignant brain tissues (19). These studies demonstrated the ability to define both tumor-specific endothelial genes and normal endothelial genes. It is noteworthy that the genes discovered to be growth or tumor-specific could be generally classified as extracellular matrix components or surface proteins likely to play a role in adhesion or cell-cell interaction. Transcription factors and other classes of upstream regulators were generally lacking. This finding suggested that the molecular events driving tumor angiogenesis were either solely dependent on extracellular events or that tumor-specific transcription factors were at too low a level to be evaluated with the expression platform used. Moreover, the extent to which circulating endothelial cells and vasculogenesis contribute to the overall expression changes is unclear. Here, we report our findings on serial analysis of gene expression analysis of purified endothelial cells from freshly resected specimens of two invasive breast cancers and one normal reduction mammoplasty. The gene expression profiles derived in our current study define unique profiles for vascular gene expression in breast tumorigenesis.

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MATERIALS AND METHODS

Immunopurification of Specific Cell Populations. Fresh resected tissue (normal mammoplasty or primary breast tumor tissue) was obtained from the Johns Hopkins Hospital Surgical Pathology Division with approval from the Institutional Review Board and processed immediately. The surgically resected samples were kept at room temperature for no more than 30 minutes after resection. Tissue was collected from surgical pathology on ice. Digestion of the minced tissue was carried out at 37°C up to a maximum of 90 minutes, depending on the speed of tissue disruption, which varied with each tissue. Tissue was digested with collagenase A at 37°C to yield a single cell suspension. This was followed by a number of negative selections including red blood cells lysis (NH_4Cl) and removal of penetrating immune cells including monocytes, lymphocytes, and macrophages (anti CD-14 and CD-45 DynaBeads, Dynal Biotech, Brown Deer, WI). Epithelial and endothelial cells were then positively selected using the Dynal magnetic bead-linked antibody method essentially as described (16, 20). Epithelial cells were removed before selection with P1H12 Dynal immunobeads. Immunostaining was then performed using von Willebrand factor and 4′, 6-diamidino-2-phenylindole nuclear staining to confirm the purification of endothelial cells. Under these conditions the secondary antibody bead binding to P1H12 surrounding the cells also allowed for additional confirmation of cell identity. Immunostaining revealed that the immunopurification technique yielded endothelial cell purity of >95% (data not shown).

Serial Analysis of Gene Expression Analysis. RNA was extracted from ~20,000 purified cells. LongSAGE analysis (21) was performed on RNA from the endothelium of 2 breast tumor samples and 1 normal mammoplasty tissue, yielding ~50,000 tags (Table 1). All of the tag frequency calculations are normalized to exactly 50,000 tags. For comparative analysis to the colon standard serial analysis of gene expression tag libraries, longSAGE tag counts were aggregated based on common 10 base sequence tags. Genes that corresponded to the tags were then determined, and the fold difference between tags from normal breast endothelium and breast tumor endothelium were calculated. To provide for a conservative estimate of tumor-induced and normal-induced genes, a ratio was calculated using the minimum tumor serial analysis of gene expression tag number (for tumor-induced to normal-induced ratios) or the maximal tumor serial analysis of gene expression tag number (for normal-induced to tumor-induced ratios). Data derived from normal brain endothelial cells and glioma endothelial cells were used as comparators to define breast-specific markers (19).

***In situ* Hybridization/Immunohistochemistry.** For *in situ* hybridization, a mixture of riboprobes for each gene was generated yielding products of 450 to 550 bases as described (16). Briefly, riboprobes were generated from an amplification of the DNA sequence of interest, involving incorporation of a T7 primer in an initial PCR reaction followed by use of T7 polymerase for *in vitro* transcription. The *in vitro* transcription also included labeling of transcripts with digoxigenin (Roche Diagnostics, Indianapolis, IN). Paraffin-embedded sections of normal breast tissue, primary breast cancers of various stages, and tissue arrays were obtained from the Johns Hopkins Hospital Surgical Pathology Division and used for *in situ* hybridization and immunohistochemistry. For *in situ* hybridization, individual riboprobes were initially used to determine those that gave the best signal with minimal background DNA binding, and these were pooled into a mixture to be used for all of the subsequent hybridizations for each gene studied. Protocols were as described previously, including deparaffinization and fixation of tissues and pretreatment for access to target nucleic acid sequence, followed by addition of digoxigenin-labeled riboprobe overnight (16). Addition of horseradish peroxidase-anti-digoxigenin (Dako) and two rounds of tyramide amplification involved the addition of biotinyl tyramide (Dako) that binds in the vicinity of horseradish peroxidase. Signal was visualized using the 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium alkaline phosphatase system (involving the addition of a Dako AP-antibiotin). Some were counterstained with nuclear fast red.

***In vitro* Functional Assays.** Adenovirus expressing HEYL and PRL3 were constructed and determined to viably infect human microvascular endothelial cells. Both constructs expressed the gene of interest to a level at least 15-fold higher than empty vector control. The effect of HEYL overexpression on human microvascular endothelial cell proliferation was assessed by infecting human microvascular endothelial cells for 18 hours at a multiplicity of infection of 200. Cell number was determined at days 0, 2, and 3. The migration effects of adenoviral-expressing PRL3 were assessed by infecting 50,000 human microvascular endothelial cells for 48 hours with a multiplicity of infection of 300. Cells were plated in the top well containing basal media and migrated toward the bottom chamber containing 5% fetal bovine serum as attractant. Migrated cells were read after 24 hours using cell titer glow (Promega, Madison, WI). Terminal deoxynucleotidyl transferase-mediated nick end labeling assays were used to assess the apoptotic potential of human umbilical vein endothelial cells infected with HEYL or control adenovirus. Twenty-five thousand human umbilical vein endothelial cells were transfected with AD-GFP or AD-HEYL and cultured for 3 days in serum-free medium. Apoptosis was detected using *In Situ* Cell Death terminal deoxynucleotidyl transferase-mediated nick end labeling detection kit, TMR red (Roche).

Quantitative PCR. ABI Prism 7900 detection system was used according to the manufacturer's specifications. Data output is shown as log2 values relative to the mean of the 10 normal breast tissue RNAs used. Data were normalized to either 18 s rRNA or to the median of a set of preselected ubiquitous endothelial cell marker genes. These genes were defined as those having small variance in previously derived pure endothelial cell serial analysis of gene expression data. The genes included in the set are: *Claudin 5, ROBO4, TIE2, Hevin, Sox18,* and *von Willebrand factor.* Gene-specific PCRs were performed in duplicate on two separate occasions with similar results. Probe sequences used for gene-specific amplification were as follows: KDR: TCCCAGGCTGCACCCATGGC, TEM7: CTTTGCCTATAAAGAGATCCCTATGTCTGTCCCGG, PRL3: TTTGACGATGGGGCGCCCCCGC, HEYL: ACGGCGTCGAGACCGCATCA, Neuritin: CCGCAGGGCCTGGACGACAA, and TEM1: CGCTGGCTGTCGACGGCTACCTGTGCCAGTT.

PRL3 Expression in Serial Analysis of Gene Expression Libraries. PRL3 expression (serial analysis of gene expression tag = taggtcagga) was determined for breast-specific cells or tissue in both public serial analysis of

Table 1 *Sample summary*

A. Endothelial samples used for SAGE analysis

Sample	Disease	Identity	Cell number	SAGE tags
Endo 1	Tumor	BEC T1	30,000	51,000
Endo 2	Tumor	BEC T2	20,000	52,000
Endo 3	Normal	BEC N1	40,000	52,000

B. Confirmation of cell identity

Gene description	UG ID	Specificity	BEC T1	BEC T2	BEC N1	HF	MDA-453
von Willebrand factor	Hs.110802	EC	15	26	16	0	0
Platelet/endothelial cell adhesion molecule (CD31)	Hs.78146	EC	13	25	25	8	0
CD34 antigen	Hs.367690	EC	6	15	26	1	0
SPARC-like 1 (mast9, hevin)	Hs.75445	EC	113	117	121	0	0
Keratin 8	Hs.242463	Epithelial	1	0	0	4	124
CD14	Hs.163867	Hematopoeitic	0	0	0	50	0
CD45	Hs.444324	Hematopoeitic	0	0	0	7	0

NOTE. In B, the number of tags for genes expressed in specific cell types are shown in the tumor endothelial samples and in the normal breast endothelial cells. A hematopoietic SAGE library (HF) and breast cancer cell line SAGE library (MDA-453) are also included. All tag frequencies are per 50,000.

Abbreviations: SAGE, serial analysis of gene expression; EC, endothelial cell.

gene expression database resources (CGAP) and our own serial analysis of gene expression database. Library data from our database included B1 and B2; normal bronchial epithelial cells; and tumor cell lines B5, B6, B7, B8, B9, and B10. Additional tumor lines included 21-PT, 21-MT, MDA-467, SKBR3, BT-474, and MDA-231; BEC1: BECT1, BEC2: BECT2 and BEC3; BECN1 (this study); BEC5; bone metastasis epithelial cells, BEC6; and PCR amplified normal breast epithelial cells. All of the other library information can be found on the internet.[3]

RESULTS

Data Generation and Confirmation of Cell Purity. We performed longSAGE analysis on endothelial cells isolated from 1 normal breast sample and 2 invasive ductal carcinoma samples. A summary of the samples used, the approximate number of endothelial cells purified, and the serial analysis of gene expression tags accumulated are summarized in Table 1*A*. Each library was sequenced to just over 50,000 tags. All of the additional tag calculations are based on normalization of tag counts to exactly 50,000. Epithelial cell isolates were also collected but used solely to define relative cell specificity by PCR (see below).

The premise for our study is that pure endothelial cell populations can be derived from surgical samples of both normal and malignant breast tissue. The protocol used for the isolation of pure endothelial cells uses a combination of negative and positive immunoselections. Immunopurification of specific cell types requires evidence that the final cell preparations are essentially pure. We confirmed the purity of our endothelial cell populations by analyzing the serial analysis of gene expression tag frequency for genes known to be selectively expressed in specific subpopulations of cells. Genes specific for endothelial, epithelial, and hematopoietic cells were analyzed in the serial analysis of gene expression libraries for the 3 samples (Table 1*B*). Genes with preferential expression in vascular endothelium including *von Willebrand factor, CD31, CD34,* and *hevin* were moderately and uniformly expressed in all 3 of the samples. Genes specific to epithelium (*keratin 8* and *6A*) or hematopoietic cells (CD14 and CD45) had negligible expression within the constructed serial analysis of gene expression libraries with the total absence of corresponding tags in most cases. Furthermore, von Willebrand factor and CK18 reverse transcription-PCR analysis performed on cDNA generated from the endothelial cell populations demonstrated little contamination of epithelial cells within the endothelial cell populations (data not shown). This confirms that the samples used were essentially pure endothelial cells. However, it is reasonable to assume that a small percentage of cells are derived from endothelial cell-associated pericytes.

Genes Expressed in Invasive Breast Cancer Endothelium. Genes that are overexpressed in cancer offer attractive therapeutic targets, especially if expressed at relatively low levels in normal tissues. Table 2*A* represents genes that were expressed at least 6-fold higher (conservatively calculated by taking the tag ratio of the lower-expressing tumor sample over the tag frequency in the normal sample) in breast tumor endothelium when compared with the endothelial cells isolated from normal mammoplasty tissue. Table 2*A* also shows the tumor induction ratio of the genes in a previously examined colon tumor study (16).

As expected, genes encoding proteins involved in extracellular matrix function (collagens, MMP9, ADAMTS4, and TIMP1) were transcriptionally active in tumor vasculature compared with normal breast vasculature (Table 2*A*). Most of these extracellular matrix-encoded genes were similarly up-regulated in colon tumor vasculature (Table 2*A*). Unexpectedly, we identified the transcription factors SNAIL1 and HEYL as being induced in breast tumor vasculature. Although HEYL was also observed to be transcriptionally up-regulated in colon tumor vasculature (tag: ggttgttgcg), the induction relative to normal colon vasculature was moderate. In contrast, both SNAIL1 and HEYL were induced at least 10-fold in breast tumor vasculature relative to normal breast vasculature. Five genes could be recognized as being induced $\geq$10-fold in breast tumor endothelial cells and induced at least 5-fold higher in breast than that observed in both colon and brain tumor endothelial cells (see serial analysis of gene expression analysis in Materials and Methods; Table 2*B*). Two of these five genes included *SNAIL1* and *HEYL*.

Also unexpected was the finding that PRL3 was highly induced in breast tumor vasculature, because this gene was shown previously to be expressed primarily in epithelial tumor cells (17). It is noteworthy that although both HEYL and PRL3 serial analysis of gene expression tags demonstrate a differential induction in tumor endothelial cells over normal endothelial cells, the specific tag within the genes that is induced differs between colon and breast endothelial cell libraries (Table 2*C*). For both HEYL and PRL3, the most differential tag frequencies observed for colon endothelial cells derives from a 3′ extended form of the transcripts. These extended transcripts are based on gene prediction algorithms incorporating all of the available expressed sequence tag data. Thus, within the colon endothelial cell data, the recognized 3′ ultimate tags for HEYL and PRL3 show limited or no tumor induction, respectively. It remains unclear why there is this differential transcript detection between colon and breast endothelial cells.

Finally, the robust induction of the cell-cell interaction protein VE-cadherin was unique to breast tumor vasculature, with no induction in colon tumor vasculature (Table 2*A*) or brain tumor vasculature (data not shown).

Decreased Gene Expression in Invasive Breast Cancer. The concerted reduction or absence of expression of genes in tumor vasculature as compared with normal vasculature may reveal genes that function to suppress tumor and/or vascular growth. With this in mind, we sorted the data to reveal genes that showed little or no expression in tumor vasculature compared with normal breast vasculature. A striking down-regulation of numerous genes was observed (Table 3). Genes expressing secreted proteins may lend themselves to direct therapeutic intervention and are likely to play a role in extracellular matrix stabilization or cell adhesion. Particularly noteworthy is the observed down-regulation of both lysyl oxidase-like 1 and lysyl oxidase. Members of the lysyl oxidase gene family have been implicated in the regulation of tumor growth, albeit with highly contrasting results from different studies (22). The involvement of lysyl oxidase genes in extracellular matrix formation and repair may have implication for regulating the plasticity of tumor vasculature (23).

We were interested in learning which tumor-repressed, vascular genes were conserved in their reduced gene expression across different tumor types. The most highly conserved gene down-regulated in tumor vasculature is the neuritin gene (NRN1 and CPG15), exhibiting a 9-fold reduction in breast tumors (Table 3) and a 6- and 4-fold reduction in colon and glioma tumors, respectively (data not shown). Neuritin encodes a glycosylphosphatidylinositol-anchored protein that has been demonstrated to affect neurite growth *in vitro* (24).

Confirmation of Gene Expression Alteration in Normal, Ductal Carcinoma *In situ*, and Invasive Ductal Carcinoma Tissue. The expression patterns for selected genes observed in the serial analysis of gene expression library were confirmed by reverse transcription-PCR (Fig. 1*A*). In addition to the purification of endothelial cells from normal and tumor breast tissue, we also immunopurified the adjacent epithelial cells. RNA derived from both endothelial cells and epithe-

[3] Internet address: http://www.ncbi.nlm.nih.gov/SAGE/index.cgi?cmd = libsearch.

Table 2 *Tumor selective genes*

A. Breast tumor-vascular genes

Long Tag	BEC T2	BEC T1	BEC N1	Breast T/N	Colon T/N	UG ID	Description
TAAATCCCCACTGGGAC	27	21	0	21	3	Hs.151738	Matrix metalloproteinase 9 (gelatinase B)
GGTTGTTGCGGACATCC	31	20	0	20	3	Hs.23823	Hairy/enhancer-of-split HEYL
CCACCGGATTCTCCTCC	16	15	1	15	4	Hs.377812	Homo sapiens clone FLC1492 PRO3121 mRNA
AACACAGCCTGGGACCA	13	13	1	13	0	Hs.278625	Complement component 4A
ACAGACTGTTAGCCAAG	20	12	1	12	18	Hs.125036	Tumor endothelial marker 7 precursor
CCCATTTCTGTGGAGCC	11	10	0	10	0	Hs.48029	Snail homolog 1 (*Drosophila*)
GAGCTGGCATAACATTG	9	13	1	9	2	Hs.394790	Collagen, type IV, α2
CAGAGATGAATTTATAC	17	45	2	9	11	Hs.8997	Heat shock 70kDa protein 1A
TTACTCTCCTATTTTCA	18	17	2	9	2	Hs.111779	Secreted protein, acidic, (osteonectin)
TAATCCTCAAGAAATAA	16	24	2	9	4	Hs.78409	Collagen, type XVIII, α 1
TAGGTCAGGACCTTGGC	20	16	2	8	1	Hs.43666	Protein tyrosine phosphatase type IVA, member 3 (PRL3)
AATCTCCCCCTCCCGCG	46	24	3	8	1	Hs.833	Interferon-stimulated protein, 15 kDa
GAGAGTGTCTGCGGATA	30	22	3	7	3	Hs.5831	Tissue inhibitor of metalloproteinase 1
GGTGGACACGGATCTGC	7	7	1	7	2	Hs.111779	Secreted protein, acidic, cysteine-rich (osteonect)
AATAAACGCTACACGCT	7	8	1	7	1	Hs.179735	Ras homolog gene family, member C
TACTAGTCCTCTAGAAA	7	8	1	7	5	Hs.289088	Heat shock 90kDa protein 1, α
GAGTAACGCGCCCGGCC	9	7	0	7	1	Hs.122359	Calcium channel, voltage-dependent, α 1H
TTTCTTAAAGCCATTTT	7	11	0	7	1	Hs.197114	Serine/arginine repetitive matrix 2
GTTGTAAAATAAACTTT	7	9	0	7	1	Hs.7869	ESTs, Highly similar to PLCD_HUMAN 1-acyl-sn-glyce
GGCAGCCAGAGCTCCAA	7	8	0	7	2	Hs.75061	Macrophage myristoylated alanine-rich C kinase
TATGAGGGTAATCGACA	13	12	2	6	2	Hs.24950	Regulator of G-protein signaling 5
CATAATTTTTGTTCTTC	6	6	1	6	1	Hs.6468	HSPC142 protein
CTGTCCTAGCAGCCACC	12	13	2	6	0	Hs.12956	Tax interaction protein 1
ACAGGGTGGGAGGGCCC	12	15	2	6	5	Hs.82646	DnaJ (Hsp40) homolog, subfamily B, member 1
AAGTACGACGAAAACTT	6	10	1	6	1	Hs.380824	ESTs, Weakly similar to S55016 protein oaf - fruit
TGAAATAAAACTCAGTA	6	11	1	6	1	Hs.9614	Nucleophosmin (nucleolar phosphoprotein B23)
ACAAGTACTGTATTTTT	6	17	1	6	1	Hs.76206	Cadherin 5, type 2, VE-cadherin
TTCTCCCAAATACCCTT	71	69	12	6	4	Hs.394790	Collagen, type IV, alpha 2
TTCACAGATTTGAAAGA	6	6	0	6	1	Hs.408936	G-protein γ-12 subunit
TAAAAATGTTTCAAAAA	6	7	0	6	2	Hs.82085	Serine (or cysteine) proteinase inhibitor, clade E

B. Breast-specific, tumor vasculature genes

Short Tag	Colon T/N	Brain T/N	Breast T/N	Unigene ID	Description
GAGCTGGCAT	2	2	11	Hs.75617	Collagen, type IV, alpha 2
AACACAGCCT	1	1	13	Hs.170250	Complement component 4A
GCCATTTCTG	0	1	10	Hs.48029	Snail 1 (drosophila homolog)
CGTTGTTGCG	3	0	20	Hs.23823	Hairy/enhancer-of-split HEYL
TTTGTTAAAA	1	0	10	Hs.78054	Pre-mRNA splicing factor similar to *S. cerevisiae*

C. PRL3 and HEYL observed tag variants

	BEC2 (T)	BEC1 (T)	BEC3 (N)	Colon normal	Colon tumor
PRL3					
taggtcagga	20	16	2	4	4
ttgggtgaaa (3′ extended)	0	0	0	0	4
HEYL					
ggttgttgcg	31	20	0	3	10
cctggttcag (3′ extended)	0	3	0	0	7

NOTE. In A, genes upregulated ≥6-fold in breast tumor ECs relative to normal breast ECs. In B, genes demonstrating both a 10-fold induction in breast tumor vasculature and a >5-fold induction than observed in colon or brain tumor vasculature. In C, tag frequency for PRL3 and HEYL tag variants observed in breast EC data and colon EC data.

Abbreviations: EC, endothelial cell; EST, expressed sequence tag; T, tumor-induced; N, normal-induced.

lial cells were tested for expression of SNAIL1, HEYL, PRL3, and VE-cadherin. As expected, SNAIL1 was expressed in both the endothelium and the epithelium of invasive cancer. The expression of SNAIL1 in tumor epithelium was reported previously, but its expression in breast cancer endothelium is a novel finding (25, 26). HEYL and PRL3 expression was negligible in both cell types in normal mammoplasty tissue. In invasive breast cancer, the expression of HEYL and PRL3 was observed predominantly in the endothelial cell population, with relatively minimal expression in tumor epithelial cells. As noted previously, VE-cadherin was seen to be expressed at low levels in the endothelium of normal breast tissue. This was also the case using reverse transcription-PCR where expression was detectable in normal endothelium but was present at significantly higher levels in the tumor endothelium (Fig. 1*A*).

We additionally validated the observed overexpression of selected genes by performing quantitative real-time PCR on a panel of normal breast tissue ($n = 10$) and breast tumor tissue RNAs ($n = 20$; Fig. 1*B*). The tumor vasculature-induced genes *PRL3* and *TEM7* showed elevated expression (>2 fold) in 63% and 37% of the tumors studied, respectively, when the data were normalized to vascular content within the tissue samples (see Materials and Methods). The prevalence of PRL3 overexpression in breast tumor tissues was reduced to 40% of the tumors when expression was normalized to 18 s rRNA, suggesting that there is contribution to PRL3 expression from nonvascular sources (data not shown). Of the genes tested, the *HEYL* gene showed the most dramatic induction in breast tumor vasculature, demonstrating an 83% tumor induction (Fig. 1*B*). We also confirmed a pattern of tumor down-regulation of neuritin in breast cancer (46% of tumors <2-fold down-regulated), consistent with our primary expression data (Fig. 1*B*). Neither TEM1 nor KDR demonstrated a robust induction in breast tumor samples relative to normal breast tissue controls.

As an extensive database of breast serial analysis of gene expression data exists both from our work and within the pubic domain, we determined the expression of PRL3 across all of the breast serial analysis of gene expression studies to date. Expression of PRL3 is clearly elevated in tumor endothelial cells relative to normal endothelial cells, bulk tissue samples, and breast tumor cell lines (Fig. 1*C*),

Table 3 *Breast normal-specific vascular genes*

Sequence	BEC T2	BEC T1	BEC N1	N/maxT	UG ID	Description
CCCGCCAGTGCACAGGG	1	0	23	24	Hs.256297	Integrin, α 11
CACCTCTCATACCCAGG	0	0	17	17	Hs.65436	Lysyl oxidase-like 1
CCACGTGGGCTCATATG	3	1	42	15	Hs.179665	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)
CCCTACCCTGTTACCTT	1	1	13	13	Hs.75736	Apolipoprotein D
GTACTACTCTTTTTTAC	4	3	42	11	Hs.303649	Chemokine (C-C motif) ligand 2
CAGTCTGAAGATTTTGC	0	0	11	11	Hs.306357	Immunoglobulin κ constant
CTCCCCCAAATCCAGAG	0	0	11	11	Hs.103291	Neuritin 1
CTCTCAGCCCCCACTAG	0	0	11	11	Hs.74602	Aquaporin 1 (channel-forming integral protein, 28k)
GCCAGGTGGCCACTCCA	1	0	10	10	Hs.111301	Matrix metalloproteinase 2 (gelatinase A, 72kDa ge
CATCTTCTAAGATTGCC	0	0	10	10	Hs.356487	ESTs, weakly similar to cytokine receptor-like fa
GGCTCCCCTGGGCAGCC	2	2	15	8	Hs.74566	Dihydropyrimidinase-like 3
GAGCTCAGTGGAACTTA	2	2	15	8	Hs.374990	CD34 antigen
CCGTGACTCTGGACTAT	1	1	8	8	Hs.296267	Follistatin-like 1
CTCTTCTGCACTTTGCT	0	0	8	8	Hs.105689	MSTP031 protein
AGCCACTGTGTCTGGCC	0	0	8	8	Hs.147313	Similar to CMRF35 antigen precursor (CMRF-35)
TCGAAGATTCACTGGGA	1	0	7	7	Hs.103395	Hypothetical protein FLJ14146
AAACGCATCAGTCCCCC	1	0	7	7	Hs.256297	Integrin, α 11
CCAGTAACCCCAGCTAC	1	0	7	7	Hs.115175	Sterile α motif and leucine zipper containing
CCCCCCTGTCCAGCGAG	1	1	7	7	Hs.381214	Hypothetical protein DKFZp434N0650
GCCTCTACAACCTCAAA	1	2	13	7	Hs.162	Insulin-like growth factor binding protein 2, 36kD
GGAACACACAGCACAGA	0	0	7	7	Hs.286049	Phosphoserine aminotransferase
AGACAGTCATTTTTAAC	0	0	7	7	Hs.17109	Integral membrane protein 2A
ACCCTCAACAGCAGAAA	0	0	7	7	Hs.119	Wilms' tumour 1-associating protein
AAGGAGAACTGAGTGAC	0	0	7	7	Hs.20144	Chemokine (C-C motif) ligand 15
GACTGCGCGTCTTGCAG	2	0	13	7	Hs.10086	Type I transmembrane protein Fn14
CTGCCGTGACAACAGCC	1	0	6	6	Hs.75462	BTG family, member 2
CAGAACAACTATCTTTG	1	0	6	6	Hs.77858	Mesenchyme homeo box 2 (growth arrest-specific hom
CACGGAGGCGGGGTCAG	1	0	6	6	Hs.320	*Xeroderma pigmentosum* complementation group C
AATAAATTCCTTCAACG	1	0	6	6	Hs.76307	Neuroblastoma, suppression of tumorigenicity 1
CCGCTGATCCACTCTCA	1	0	6	6	Hs.184161	Exostoses (multiple) 1
CGTATGCCTCAGGCTGC	1	0	6	6	Hs.102267	Lysyl oxidase
GACCTATCTCTATTGTA	1	0	6	6	Hs.194431	Palladin
GCTGCTGCGCGCGGGCT	1	0	6	6	Hs.8102	Ribosomal protein S20
TTCAGGAGGGGCTGGAT	1	0	6	6	Hs.5890	Hypothetical protein FLJ23306
GAAGTTGCCTTATTGTC	1	1	6	6	Hs.26777	KIAA0843 protein
TTGATCGAAGTGACTTT	1	1	6	6	Hs.49007	Poly(A) polymerase α
TTTCCTGTTTAGATAAG	1	1	6	6	Hs.99210	ESTs, weakly similar to hypothetical protein FLJ2
ACTCCCTCCTTAAAAAG	1	1	6	6	Hs.76230	Ribosomal protein S10
ACTATTTAAAGCCAGGG	1	1	6	6	Hs.63908	ORM1-like 2 (S. cerevisiae)
GTCTGCTCCAGGAGCTG	1	2	12	6	Hs.32978	Proprotein convertase subtilisin/kexin type 7
CAGCAAGAAGTCCTTGC	1	2	12	6	Hs.322704	ESTs, weakly similar to T02670 probable thromboxan
GTTCTCCCACTGCCCTG	0	1	6	6	Hs.306079	Protein transport protein SEC61 α subunit is of
TCATTGGCCTTCAGATG	0	1	6	6	Hs.75777	Transgelin
TAGGTCAACAAACTTGC	0	1	6	6	Hs.183434	ATPase, H+ transporting, lysosomal interacting pro
TAACCGCAGATAGCAAG	0	1	6	6	Hs.74376	Olfactomedin 1
TTTCATTTGCTCAGTCG	0	1	6	6	Hs.132821	EST, highly similar to FM02_HUMAN DIMETHYLANILINE
ACACTTCTTTCAGTATA	0	1	6	6	Hs.83381	Quanine nucleotide binding protein 11
CTGGAAACGCCGGAACC	0	1	6	6	Hs.1480	Histidine rich calcium binding protein
GACAGTTGTTTGCAGAA	0	1	6	6	Hs.760	GATA binding protein 2
GCCCACGTAGCCCCACT	0	0	6	6	Hs.155597	D component of complement (adipsin)
AAGGATGCGGTAATGGC	0	0	6	6	Hs.83126	TAF11 RNA polymerase II, TATA box binding protein
GCCAGACCCCTCCCACC	0	0	6	6	Hs.306193	Hypothetical protein LOFBS-1
TTCTGCAAGACTACTTG	0	0	6	6	Hs.75462	BTG family, member 2
CAAGCGTAAGAATGACT	0	0	6	6	Hs.76224	EGF-containing fibulin-like extracellular matrix p
CAGTCGCTTCAGTGCAA	0	0	6	6	Hs.8302	Four and a half LIM domains 2

NOTE. Genes upregulated ≥6-fold in normal breast ECs relative to breast tumor ECs.
Abbreviations: EC, endothelial cell; EST, expressed sequence tag.

lending additional support to a preferential expression of PRL3 in breast tumor vasculature relative to epithelial cells. The PRL3 tag was observed at a frequency of 11 tags to 50,000 in one public normal breast endothelial cell library (Br-N-Endothelial). However, these data were excluded from Fig. 1*C*, because it was observed that this library showed significant expression of both epithelial and hematopoietic-specific genes, in obvious contrast to other fresh tissue-derived endothelial cell libraries.

In situ hybridization was used to localize the expression of gene transcripts in tumor vasculature. For all of the *in situ* hybridization experiments, KDR (VEGFR2) was used as a control to localize the endothelial cells within the vasculature. This allowed confirmation of vascular-specific staining with the probes of interest. A HEYL mixture was used to probe for the expression of HEYL in normal tissue and tissue derived from a patient sample of invasive ductal carcinoma (Fig. 2). When comparing staining in the normal tissue with that of H&E and KDR, it is evident that the HEYL probe does not stain the endothelium in normal breast tissue vasculature. In contrast, the invasive breast cancer section demonstrates strong labeling by the HEYL-specific probes paralleling the staining seen with the endothelial-specific marker KDR. Additional interrogation of a human breast tissue array demonstrates conclusive labeling of HEYL within invasive carcinoma (data not shown).

A similar trend was also observed when *in situ* hybridization was performed using a mixture of PRL3 riboprobes. Similar to HEYL, there was an absence of PRL3 RNA expression in the normal tissues analyzed, whereas clear, endothelial-specific expression was observed in the blood vessels of invasive carcinoma (Fig. 2). It is important to note that expression appeared to be limited to vascular cells with a lack of binding to the epithelium or other stromal components. There

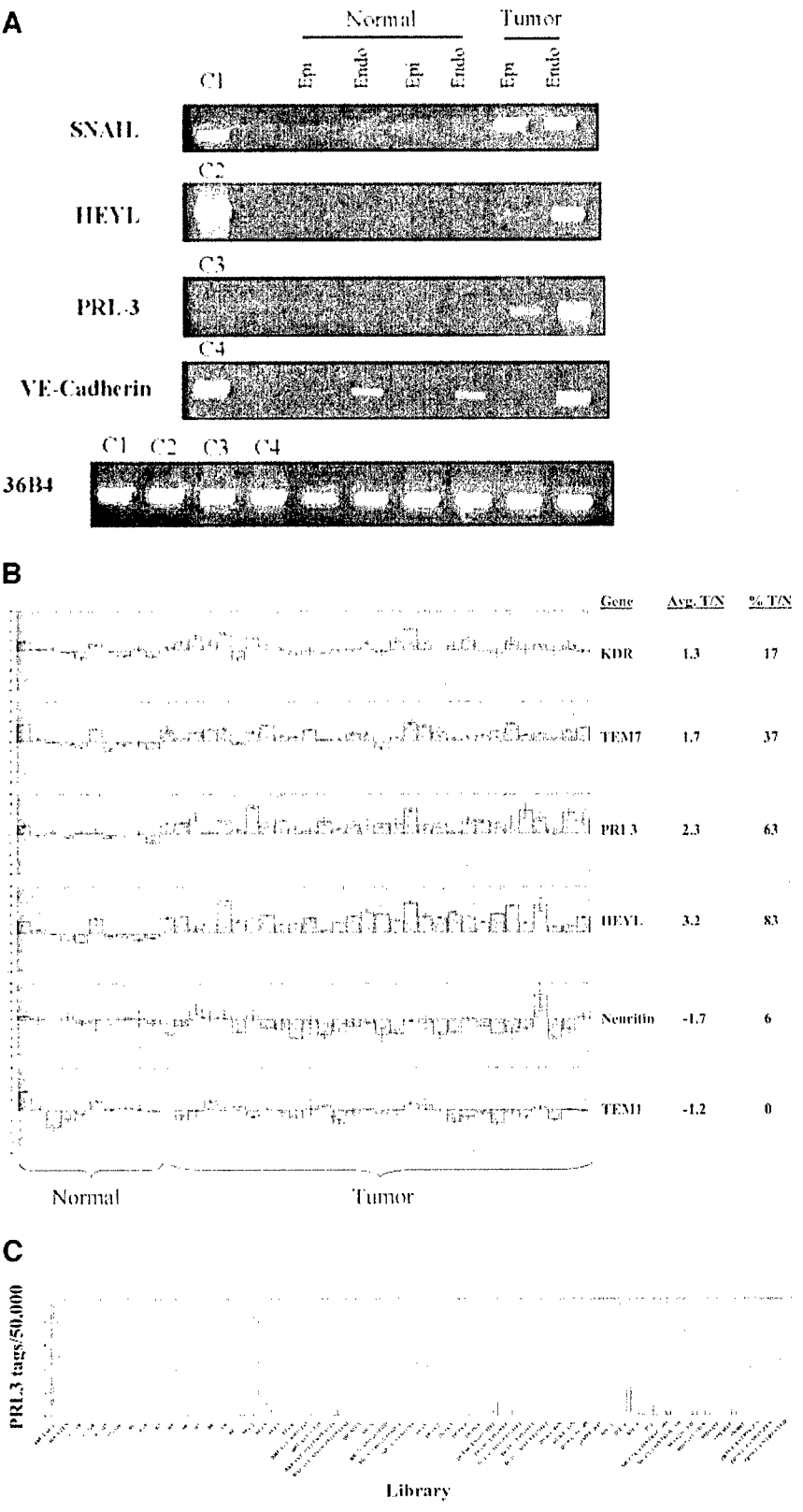

Fig. 1. Expression of selected breast tumor vascular markers. *A*, RNA from cells immunopurified from normal breast tissue and that from invasive breast cancer was reverse transcribed. Reverse transcription-PCR was then performed using primers specific for SNAIL1, HEYL, PRL3, and VE-cadherin. The 36B4 gene (ribosomal protein) was amplified as a control that should be expressed in all samples. *B*, quantitative real-time PCR for KDR, TEM7, PRL3, HEYL, Neuritin, and TEM1 in a panel of normal and invasive ductal carcinoma breast tumor samples. Expression was normalized to a set of epithelial cell-specific markers as described in Materials and Methods. The values are log2 representations relative to the mean of the normal samples. Normal breast tissue RNA (tissue 1 to 10), invasive ductal carcinoma breast tissue (tissues 11 to 40). Values are shown for average tumor to normal >2 fold (*Avg. T/N*) and percent tumor over normal >2 fold (*% T/N*). *C*, PRL3 expression in serial analysis of gene expression libraries. Abundance of the PRL3 serial analysis of gene expression tag was determined for in-house and public breast serial analysis of gene expression data using the tag taggteagga. Library data from our database included B1 and B2; normal bronchial epithelial cells; and tumor cell lines B5, B6, B7, B8, B9, B10. Additional tumor lines included 21-PT, 21-MT, MDA-467, SKBR3, BT-474, and MDA-231; BEC1: BECT1, BEC2: BECT2 and BEC3; BECN1 (this study); BEC5; bone metastasis epithelial cells, BEC6; and PCR amplified normal breast epithelial cells. All other library information can be found on the internet.[3]

was also a lack of binding to endothelium surrounding areas of ductal carcinoma *in situ*, indicating that these two genes, *HEYL* and *PRL3*, are only expressed in invasive carcinoma (data not shown).

In situ hybridization analysis for VE-cadherin showed a light staining in normal tissues that was enhanced in invasive carcinoma (Fig. 3*A*). Because a comparison between staining of different tissues is not quantitative, this was additionally analyzed by using a single patient sample that contained areas of normal tissue, ductal carcinoma *in situ*, and invasive ductal carcinoma on the same section. Fig. 3*B* illustrates VE-cadherin, staining as slightly positive in the normal area, becom-

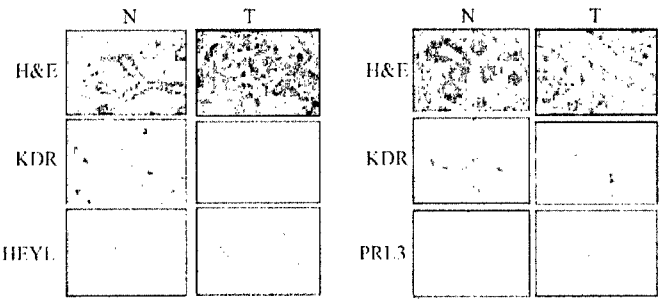

Fig. 2. *In situ* hybridization of HEYL and PRL3. *in situ* hybridization of the *HEYL* and *PRL3* genes were performed on sections of normal breast tissue (*N*) and a sample of invasive breast cancer (*T*). Serial sections were H&E stained, and *in situ* hybridization was also carried out using the endothelial-specific KDR riboprobe as a control. The *purple* staining in KDR probed sections represents the position of the endothelial cells (vasculature). ×40 magnification.

ing stronger in the vasculature surrounding the ductal carcinoma *in situ* and strongest in areas of invasive ductal carcinoma, mimicking the exact pattern of both KDR and CD31 binding. Therefore, VE-cadherin expression increases as the tumor becomes more invasive. The rim of microvessels around ductal carcinoma *in situ* is characteristic of high-grade ductal carcinoma *in situ*.

Immunohistochemical Analysis of Osteonectin Expression. Because there was a high-affinity, paraffin-reactive antibody to osteonectin available, immunohistochemistry was used to confirm the enhanced expression of this gene in breast tumor vasculature. Fig. 4 shows the results of two different cases containing either normal and invasive breast cancer or ductal carcinoma *in situ* and invasive ductal carcinoma on the same paraffin-embedded section of two patient samples. As observed with PRL3 and HEYL, there was an absence of expression (in this case protein expression) in normal tissue and ductal carcinoma *in situ*, whereas strong staining was observed in the endothelium and stromal fibroblasts of invasive breast cancer. The staining of the vascular endothelium was confirmed by comparing staining patterns with that of CD31 antibody (data not shown). Our results suggest that osteonectin protein is expressed to progressively higher levels as the tumor progresses from ductal carcinoma *in situ* to invasive carcinoma.

This finding was pursued in greater detail by examining tissue microarrays containing breast carcinoma, hematogenous metastases, and normal breast tissue. Immunohistochemistry for osteonectin was performed, and all of the spots were scored as negative, weakly positive, moderate positive, or strong positive. Among 11 cases with benign breast lobules, the endothelium and stromal fibroblasts were completely negative in 2 cases, focally weakly positive in 5 cases, weakly positive in 2 cases, and focally moderately positive in 2 cases. In contrast, all 20 of 20 primary carcinomas are positive for osteonectin. In the 20 primary carcinomas, the endothelium and stromal fibroblasts of 9 were moderately positive, 9 were moderately to strongly positive, and 2 were strongly positive. Only in 3 of the 20 primary carcinomas were the epithelial cells themselves labeled, 1 weakly and 2 moderately. Among hematogenous metastases, staining for osteonectin was more frequent and stronger. The stroma of 11 were moderately positive, 5 were moderately to strongly positive, 2 were focally strongly positive, and 8 were strongly positive. Only in 5 of 26 metastatic carcinomas were the epithelial cells themselves labeled for osteonectin, all of which did so weakly. Thus, osteonectin expression is found primarily in the endothelial cells and stromal fibroblasts and rarely in the carcinoma cells of both invasive tumors and their hematogenous metastases. Furthermore, the expression of osteonectin is higher in hematogenous metastasis compared with invasive breast cancer.

Neuritin Expression Is Decreased in Invasive Breast Cancer. We next wanted to validate the expression of the gene found to represent the most conserved, tumor-repressed vascular gene, *NRN1*. Using a riboprobe specific for the *NRN1* RNA transcript, binding was evident in the vasculature of normal mammoplasty tissue, as verified by the binding patterns of KDR (Fig. 5). The vascular-specific binding of NRN1 probe was abolished in invasive breast cancer tissue (Fig. 5).

Functional Assessment of Breast Tumor-Induced Vascular Genes. We next addressed the functional implications of overexpressing specific breast tumor endothelial cell genes. Adenovirus expressing either HEYL or PRL3 was used to infect human microvascular endothelial cells. The transfected cells were assessed for their ability to affect migration, proliferation, and tube formation relative to

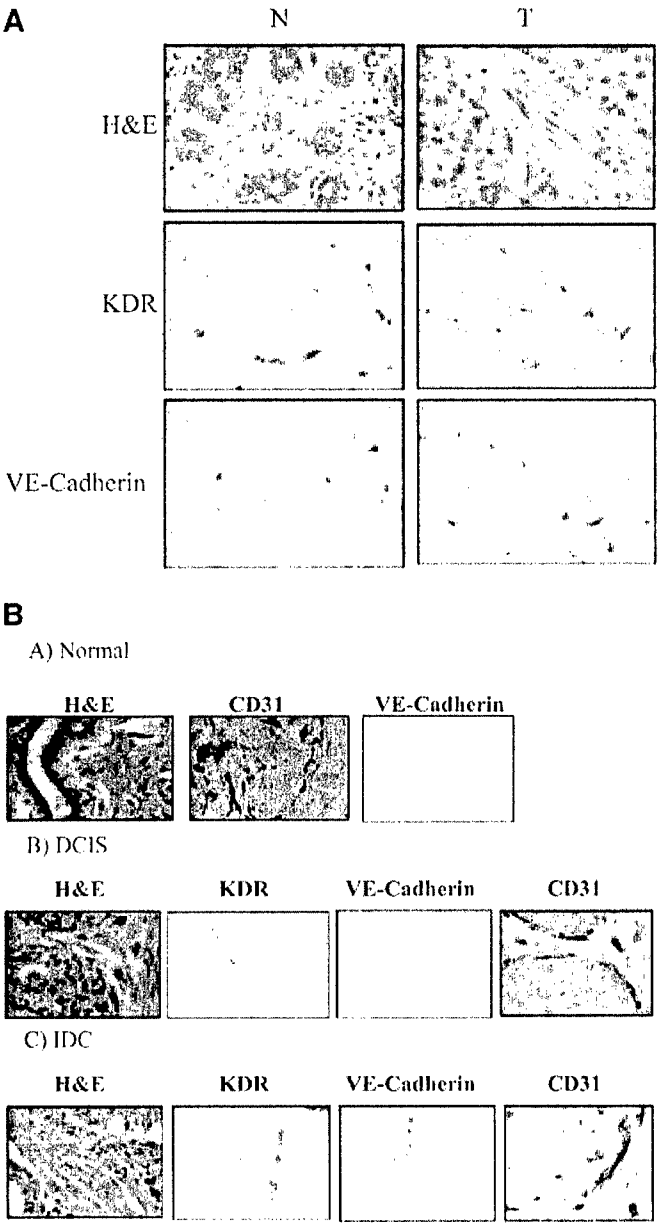

Fig. 3. *In situ* hybridizations for VE-cadherin expression. *A*, mixtures of KDR and VE-cadherin riboprobes were hybridized to a breast tissue array containing normal (*N*) and tumor (*T*) sections. Arrays were also H&E stained to determine the presence of normal or tumor epithelium and, hence, the location of tumor endothelium. *B*, a breast tumor sample that contained regions of normal breast (*A*), ductal carcinoma *in situ* (*DCIS*; *B*), and invasive ductal carcinoma (*IDC*; *C*) on the same section was stained with H&E, KDR, or VE-cadherin riboprobes or with CD31 antibodies. Results were visualized ×40.

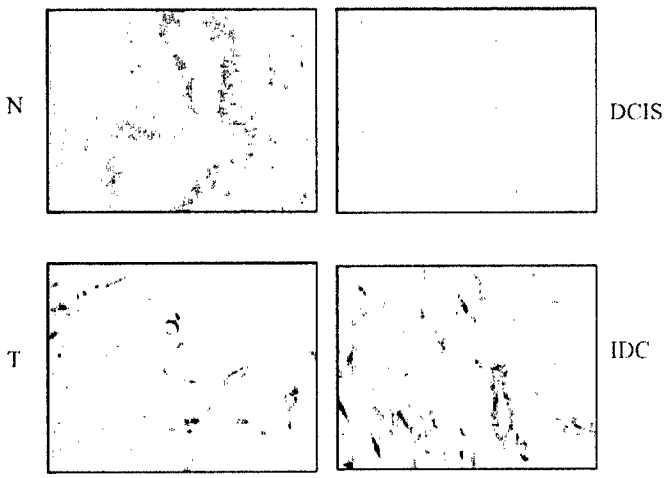

Fig. 4. Immunohistochemistry of osteonectin. An osteonectin antibody was used to probe different samples of breast cancer, one containing regions of normal breast tissue and invasive cancer on the same section (*N* and *T*) and the other having areas of ductal carcinoma *in situ* (*DCIS*) and invasive ductal carcinoma (*IDC*) on the same slide. ×40 magnification.

cells infected with empty vector or green fluorescent protein expressing adenoviral control. The overexpression of HEYL showed a clear and reproducible enhancement of human microvascular endothelial cells proliferation (Fig. 6*A*). The functional implications for HEYL overexpression were specific to cellular proliferation, as HEYL overexpression had no effect on human microvascular endothelial cell tube formation or migration (data not shown). Similar results were observed for human umbilical vein endothelial cells (data not shown). The overexpression of HEYL also showed a protective effect on human umbilical vein endothelial cell apoptosis (Fig. 6*B*). Adenovirus-expressing green fluorescent protein-infected human umbilical vein endothelial cells showed a large amount of apoptosis when cells were starved for serum. In contrast, the overexpression of HEYL resulted in healthy cells after 3 days and little apoptosis (Fig. 6*B*). Untransfected human umbilical vein endothelial cells essentially all succumbed to apoptosis (data not shown).

The overexpression of PRL3 in human microvascular endothelial cells showed an effect on cellular migration and not on proliferation (Fig. 6*C*). PRL3 overexpression had a modest effect on tube formation, increasing the quantity of tubes as compared with control-infected cells (data not shown). These results suggest separate and specific roles for individual breast tumor vascular genes.

DISCUSSION

To identify genes that may regulate tumor-directed angiogenic growth we have used serial analysis of gene expression to generate transcriptomes of endothelial cells from normal and malignant breast tissue. The work presented here demonstrates the utility of cell-specific purification linked to comprehensive gene expression analysis and expands on the limited quantitative gene expression studies performed thus far on pure, tissue-derived endothelial cells. Among the genes significantly induced in breast tumor endothelium were a number of transcription factors including HEYL and SNAIL1 and the tyrosine phosphatase PRL3, all of which potentially have numerous gene targets that may contribute to angiogenesis and tumor progression.

HEYL was identified recently as a basic helix-loop-helix transcription factor, a family of factors known as key regulators of embryonic development or differentiation (27). It has been shown that HEYL can be activated by constitutively active forms of Notch receptors, making Notch receptors upstream regulators of HEYL expression (28). The role of enhanced expression of HEYL in the breast tumor endothelium is an interesting one that needs to be studied at a functional and molecular level to determine the role of the induction of this gene on angiogenesis and tumorigenesis. The fact that HEYL and other members of the Notch pathway have limited expression in adulthood makes this gene a potential target for therapy due to the reduced likelihood of systemic toxicity.

There have been a number of publications recently regarding the role of the SNAIL1 family of zinc-finger transcription factors in tumorigenesis. Of particular interest is the role of SNAIL1 in epithelial-mesenchymal transition that could promote the invasive capacity of epithelium in breast cancer. The transition from epithelial to mesenchymal cells allows for enhanced migratory and invasive properties, and, hence, such a transition may contribute to tumor progression (reviewed in ref. 29). Our finding that SNAIL1 is induced in tumor endothelium, additional to its enhanced expression in breast tumor epithelium (26, 30), has not been reported previously and suggests a role for SNAIL1 in the transcriptional regulation of genes important in angiogenesis. Our study has demonstrated that SNAIL1 expression is enhanced 10-fold in breast tumor endothelium, whereas expression is absent in the endothelium of colon and brain carcinoma.

PRL3 is a proposed tyrosine phosphatase with a COOH-terminal prenylation motif that allows its association with the plasma membrane. This gene was reported recently to have a role in colorectal cancer metastasis (17) where it was found to be expressed at high levels in tumor cells of metastatic tumors, with a significantly lower level expression in the vasculature. Our results suggest a shift in the cell-specific expression of PRL3 in breast cancer *versus* colon cancer. We observed a 6-fold induction in breast tumor endothelium by serial analysis of gene expression yet an apparent absence or low expression in surrounding epithelium by *in situ* hybridization. There is limited information on the role of this gene and its molecular targets, yet as a phosphatase it may have important roles in cell signaling. It has been shown recently that PRL3 promotes cell motility, invasion, and metastasis of Chinese hamster ovary cells (31).

A gene found to be expressed at low levels in normal endothelium

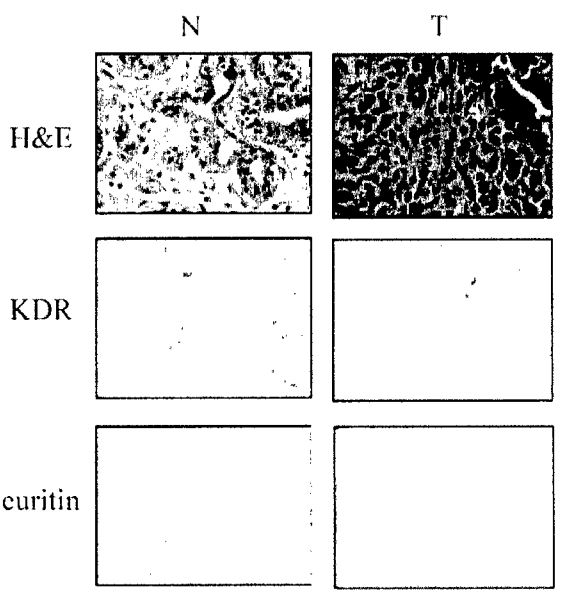

Fig. 5. *In situ* hybridization of neuritin. Serial sections from a human breast tissue array were probed with KDR as an endothelial specific control or with a mixture of riboprobes specific for the neuritin gene. Both normal samples (*N*) and invasive carcinoma (*T*) were also H&E stained to locate blood vessels.

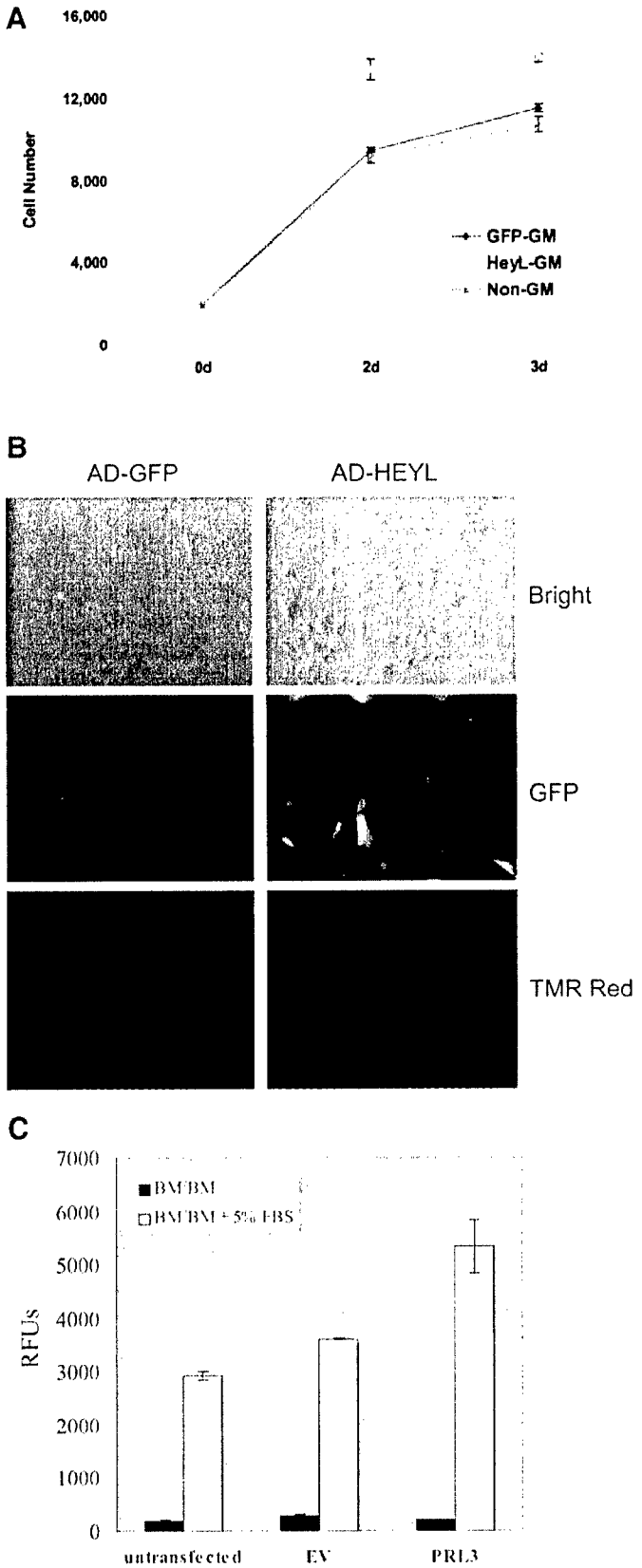

Fig. 6. *In vitro* assays on overexpressing HEYL and PRL3 endothelial cells. *A*, adenovirus expressing HEYL was used to infect human microvascular endothelial cells, and the ability of the human microvascular endothelial cells to proliferate was assessed at 0, 2, and 3 days. Green fluorescent protein-expressing adenovirus (*GFP*) or untransfected cells (*non*) were used as controls. *GM*, endothelial growth medium. *B*, terminal deoxynucleotidyl transferase-mediated nick end labeling assay detecting differential apoptosis between AD-HEYL and AD-GFP infected human umbilical vein endothelial cells. Bright field, GFP, and TMR red (apoptosis) detection fields are shown. The AD-HEYL virus used in this study also expresses GFP. *C*, adenoviral-expressing PRL3 was used to infect human microvascular endothelial cells, and the cells were assayed for migration activity. Empty vector (*EV*) or untransfected human microvascular endothelial cells were used as controls. *BM/BM*, background migration of cells in the absence of a serum stimulus. *BM/BM +5% FBS* (fetal bovine serum), cells migrating toward the serum attractant; *bars*, ±SD.

but at higher levels in breast tumor endothelium was VE-cadherin. This adherence molecule is localized to the interendothelial cell junction and has an important role in maintaining endothelial permeability. Previous studies support a role of VE-cadherin in angiogenesis and tumor growth when there is active vessel growth (32). Antibodies directed toward VE-cadherin inhibit angiogenesis and modulate endothelial permeability (33, 34). Moreover, dominant-negative mutants of VE-cadherin inhibit endothelial growth (35). Our results demonstrating an enhanced expression of VE-cadherin as disease progresses is consistent with previous findings suggesting a role for VE-cadherin in angiogenesis as opposed to vasculogenesis (reviewed in ref. 36).

For a tumor cell to invade the stroma and enter the circulation, it has to cross the extracellular matrix. This process requires proteinases (such as matrix metalloproteinase proteins) or the alteration of the extracellular matrix architecture. Osteonectin, which was found to be induced at least 7-fold in breast tumor endothelium, has a role in the latter. The fact that this protein was only expressed in endothelium of invasive breast cancer and not in ductal carcinoma *in situ* may support a role for this gene in altering extracellular matrix properties during tumor cell invasion and/or during angiogenesis. Osteonectin is a bone-matrix protein that has been previously found induced in mammary and other cancers, including prostate (37). Tumor-promoting effects seem to be specific for prostate and breast to date, as studies in ovarian cancer have conversely shown that osteonectin expression is associated with decreased endothelial proliferation and apoptotic induction (38). In normal tissue, osteonectin modulates cell-extracellular matrix interactions during tissue remodeling; regulates extracellular secretion of extracellular matrix components including regulation of the transendothelial flux of macromolecules; and is also involved in cell differentiation, cell migration, and angiogenesis. In prostate and breast cancer cells osteonectin enhances matrix metalloproteinase activity and promotes invasion and specific metastasis to bone *in vivo*. Osteonectin does not, however, stimulate tumor growth or promote invasion of cells that are not metastatic to bone (39). This is an important finding, because bone metastasis is prevalent in both types of cancer and is associated with patient mortality. Therefore, osteonectin may have a role in distant metastasis, although its expression does not have any prognostic significance in studies undertaken thus far. Past studies are limited in suggesting a role for osteonectin in breast tumor endothelium. In fact, studies have focused on the role of osteonectin expression in tumor epithelium. In contrast, our immunohistochemistry experiments using single sections as well as tissue arrays show that the endothelium is the major site of expression of osteonectin in most tumors, whereas osteonectin was detectable in a very small proportion of the carcinoma cells within a small subset of tumors. It will be of interest to determine the role of osteonectin expression in the vasculature and whether such expression can predict the metastatic outcome of breast cancer.

A number of genes with decreased expression in tumor endothelium were identified. We identified the gene *NRN1* as the most conserved, tumor-repressed vascular marker when we looked across colon, breast, and brain cancer vascular transciptomes. The neuritin gene was originally identified due to its induction by neural activity, being a downstream effector of activity-induced neurite outgrowth (40). In the context of neuronal regulation, neuritin serves to promote

growth as a membrane-bound, GPI-anchored protein (24). It has become clear recently that several gene products seem to share roles in neurogenesis and angiogenesis (41). Studies are ongoing to assess the functional significance of NRN1 expression in regulating angiogenesis.

This work provides novel insights into the genes that are altered in human breast cancer vasculature, suggesting roles in angiogenesis, tumor growth, and invasion. Although several common patterns in gene expression were observed in breast tumor vasculature compared with colon tumor vasculature, clear differences suggest unique signatures for tissue-specific tumor vasculature. Additional work will define the roles for these genes in driving tumor angiogenesis and vasculogenesis.

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REFERENCES

1. Leek RD. The prognostic role of angiogenesis in breast cancer. Anticancer Res 2001;21:4325–31.
2. Bertucci F, Viens P, Hingamp P, Nasser V, Houlgatte R, Birnbaum D. Breast cancer revisited using DNA array-based gene expression profiling. Int J Cancer 2003;103:565–71.
3. Velculescu VE, Zhang L, Vogelstein B, Kinzler KW. Serial analysis of gene expression. Science 1995;270:484–7.
4. Nacht M, Ferguson AT, Zhang W, et al. Combining serial analysis of gene expression and array technologies to identify genes differentially expressed in breast cancer. Cancer Res 1999;59:5464–70.
5. Dexter DL, Kowalski HM, Blazar BA, Fligiel Z, Vogel R, Heppner GH. Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res 1978;38:3174–81.
6. Klein CA, Blankenstein TJ, Schmidt-Kittler O, et al. Genetic heterogeneity of single disseminated tumour cells in minimal residual cancer. Lancet 2002;360:683–9.
7. Shekhar MP, Pauley R, Heppner G. Host microenvironment in breast cancer development: extracellular matrix-stromal cell contribution to neoplastic phenotype of epithelial cells in the breast. Breast Cancer Res 2003;5:130–5.
8. Kang Y, Siegel PM, Shu W, et al. A multigenic program mediating breast cancer metastasis to bone. Cancer Cell 2003;3:537–49.
9. Boudreau N, Myers C. Breast cancer-induced angiogenesis: multiple mechanisms and the role of the microenvironment. Breast Cancer Res 2003;5:140–6.
10. Haslam SZ, Woodward TL. Host microenvironment in breast cancer development: epithelial-cell-stromal-cell interactions and steroid hormone action in normal and cancerous mammary gland. Breast Cancer Res 2003;5:208–15.
11. Liotta LA, Kohn EC. The microenvironment of the tumour-host interface. Nature 2001;411:375–9.
12. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. Science 2002;296:1046–9.
13. Iacobuzio-Donahue CA, Argani P, Hempen PM, Jones J, Kern SE. The desmoplastic response to infiltrating breast carcinoma: gene expression at the site of primary invasion and implications for comparisons between tumor types. Cancer Res 2002;62:5351–7.
14. Linderholm B, Lindh B, Tavelin B, Grankvist K, Henriksson R. p53 and vascular-endothelial-growth-factor (VEGF) expression predicts outcome in 833 patients with primary breast carcinoma. Int J Cancer 2000;89:51–62.
15. Olewniczak S, Chosia M, Kwas A, Kram A, Domagala W. Angiogenesis and some prognostic parameters of invasive ductal breast carcinoma in women. Pol J Pathol 2002;53:183–8.
16. St Croix B, Rago C, Velculescu V, et al. Genes expressed in human tumor endothelium. Science 2000;289:1197–202.
17. Saha S, Bardelli A, Buckhaults P, et al. A phosphatase associated with metastasis of colorectal cancer. Science 2001;294:1343–6.
18. Porter DA, Krop IE, Nasser S, et al. A SAGE (serial analysis of gene expression) view of breast tumor progression. Cancer Res 2001;61:5697–702.
19. Madden SL, Cook BP, Nacht M, et al. Vascular gene expression in nonneoplastic and malignant brain. Am J Pathol 2004;165:601–8.
20. Bardelli A, Saha S, Sager JA, et al. PRL-3 expression in metastatic cancers. Clin Cancer Res 2003;9:5607–15.
21. Saha S, Sparks AB, Rago C, et al. Using the transcriptome to annotate the genome. Nat Biotechnol 2002;20:508–12.
22. Rost T, Pyritz V, Ratheke IO, Gorogh T, Dunne AA, Werner JA. Reduction of LOX- and LOXL2-mRNA expression in head and neck squamous cell carcinomas. Anticancer Res 2003;23:1565–73.
23. Smith-Mungo LI, Kagan HM. Lysyl oxidase: properties, regulation and multiple functions in biology. Matrix Biol 1998;16:387–98.
24. Nedivi E, Wu GY, Cline HT. Promotion of dendritic growth by CPG15, an activity-induced signaling molecule. Science 1998;281:1863–6.
25. Fujita N, Jaye DL, Kajita M, Geigerman C, Moreno CS, Wade PA. MTA3, a Mi-2/NuRD complex subunit, regulates an invasive growth pathway in breast cancer. Cell 2003;113:207–19.
26. Blanco MJ, Moreno-Bueno G, Sarrio D, et al. Correlation of Snail expression with histological grade and lymph node status in breast carcinomas. Oncogene 2002;21:3241–6.
27. Steidl C, Leimeister C, Klamt B, et al. Characterization of the human and mouse HEY1, HEY2, and HEYL genes: cloning, mapping, and mutation screening of a new bHLH gene family. Genomics 2000;66:195–203.
28. Maier MM, Gessler M. Comparative analysis of the human and mouse Hey1 promoter: Hey genes are new Notch target genes. Biochem Biophys Res Commun 2000;275:652–60.
29. Vincent-Salomon A, Thiery JP. Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. Breast Cancer Res 2003;5:101–6.
30. Cheng CW, Wu PE, Yu JC, et al. Mechanisms of inactivation of E-cadherin in breast carcinoma: modification of the two-hit hypothesis of tumor suppressor gene. Oncogene 2001;20:3814–23.
31. Zeng Q, Dong JM, Guo K, et al. PRL-3 and PRL-1 promote cell migration, invasion, and metastasis. Cancer Res 2003;63:2716–22.
32. Corada M, Zanetta L, Orsenigo F, et al. A monoclonal antibody to vascular endothelial-cadherin inhibits tumor angiogenesis without side effects on endothelial permeability. Blood 2002;100:905–11.
33. Liao F, Li Y, O'Connor W, et al. Monoclonal antibody to vascular endothelial-cadherin is a potent inhibitor of angiogenesis, tumor growth, and metastasis. Cancer Res 2000;60:6805–10.
34. Corada M, Liao F, Lindgren M, et al. Monoclonal antibodies directed to different regions of vascular endothelial cadherin extracellular domain affect adhesion and clustering of the protein and modulate endothelial permeability. Blood 2001;97:1679–84.
35. Venkiteswaran K, Xiao K, Summers S, et al. Regulation of endothelial barrier function and growth by VE-cadherin, plakoglobin, and beta-catenin. Am J Physiol Cell Physiol 2002;283:C811–21.
36. Carmeliet P and Collen D. Molecular basis of angiogenesis. Role of VEGF and VE-cadherin. Ann N Y Acad Sci 2000;902:249–62; discussion 262–4.
37. Thomas R, True LD, Bassuk JA, Lange PH, Vessella RL. Differential expression of osteonectin/SPARC during human prostate cancer progression. Clin Cancer Res 2000;6:1140–9.
38. Yiu GK, Chan WY, Ng SW, et al. SPARC (secreted protein acidic and rich in cysteine) induces apoptosis in ovarian cancer cells. Am J Pathol 2001;159:609–22.
39. Jacob K, Webber M, Benayahu D, Kleinman HK. Osteonectin promotes prostate cancer cell migration and invasion: a possible mechanism for metastasis to bone. Cancer Res 1999;59:4453–7.
40. Naeve GS, Ramakrishnan M, Kramer R, Hevroni D, Citri Y, Theill LE. Neuritin: a gene induced by neural activity and neurotrophins that promotes neuritogenesis. Proc Natl Acad Sci USA 1997;94:2648–53.
41. Bates D, Taylor GI, Minichiello J, et al. Neurovascular congruence results from a shared patterning mechanism that utilizes Semaphorin3A and Neuropilin-1. Dev Biol 2003;255:77–98.